

## A single codon insertion in the *PICALM* gene is not associated with subvalvular aortic stenosis in Newfoundland dogs

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In their original investigation Stern et al. reported an association of a single codon insertion in the *PICALM* gene (p.K599\_L600insL) with familial subvalvular aortic stenosis (SAS) in Newfoundland dogs (Stern et al. 2014). The reported findings are the basis of a commercially sold genetic test intended to help dog breeders to reduce SAS disease prevalence. In this report, we will outline that the original investigation by Stern et al. is not well designed, misinterprets data, and consequently makes incorrect claims. Additionally, we report data from a replication study that does not show any association between the *PICALM* variant and SAS.

In our opinion, the experimental data reported by Stern et al. do not support the conclusion of an association of the *PICALM* insertion with SAS. Stern et al. do not precisely

define the assumed mode of inheritance, but describe a single family of 45 dogs, which supports previous older reports of a possible dominant pattern of inheritance with variable penetrance. However, Stern et al. do not cite or discuss a published study that investigated the mode of inheritance in a large dataset of more than 200,000 Newfoundland dogs (Reist-Marti et al. 2012).

Stern et al. further report the results from a GWAS using 18 cases and 20 control dogs, which is arguably even in purebred dogs below the required cohort size to reliably detect significant associations for traits other than fully penetrant Mendelian characters. Stern et al. did not correct for the substantial population stratification in their samples and therefore report highly inflated *p* values, which might represent false positive signals. Stern et al. concede in their discussion that “a single region of interest was not readily identified by genome-wide association study” and their GWAS did not provide any meaningful positional information on a hypothetical SAS locus.

In an independent and complementary approach, Stern et al. performed an RNA-seq experiment on subvalvular ridge tissue samples from two SAS-affected Newfoundland dogs and six other non-Newfoundland dogs without SAS. They then filtered for non-synonymous variants that were exclusively present in one or both SAS-affected Newfoundland dogs and identified the *PICALM*:p.K599\_L600insL variant. This approach is not sufficient to claim a causal relationship between this variant and SAS as it should also detect many breed-specific variants. The authors do not give any descriptive statistics as to how many reads were analyzed, how many variants were detected in total, and how many variants were exclusively found in Newfoundland dogs. If the *PICALM* variant was the only private non-synonymous variant found in Newfoundland dogs, then one has to conclude that the RNA-seq experiment has

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technically failed. If there were other private non-synonymous variants in the SAS-affected Newfoundland dogs, Stern et al. did not report them.

Stern et al. then performed an association analysis of the *PICALM* variant in 26 SAS-affected Newfoundland dogs and 23 Newfoundland controls. It is not described whether these dogs were partially overlapping with the cohort used for the GWAS or with the family used for the pedigree analysis. Stern et al. found a significant association of the insertion allele with SAS (“ $p < 0.0001$ ”). Stern et al. also reported that they found the insertion in three SAS-affected dogs from other breeds, but not in 180 control dogs from different breeds.

Stern et al. further performed *in situ* histochemistry to demonstrate *PICALM* protein expression in cardiac tissues of two SAS-affected Newfoundland dogs that were either heterozygous or homozygous for the insertion. The presented figure does not provide a control that would allow to assess the specificity of the used antibody, but even more important, there are no data from the heart of a normal control dog being homozygous wildtype at the *PICALM* variant.

Finally, Stern et al. treated developing *Xenopus* embryos with a small molecule inhibitor of clathrin-mediated endocytosis and found changes in the heart outflow tract. This is arguably a very indirect experiment. The experiment does not prove that the single codon insertion has any functional effect on the *PICALM* protein and it does not prove whether this effect indeed leads to an inhibition of clathrin-mediated endocytosis, which is comparable to the one claimed to be caused by the used inhibitor. Therefore, the *Xenopus* experiment does not prove that the *PICALM*:p.K599\_L600insL variant is causally involved in the development of SAS.

As the argumentation by Stern et al. for the claimed role of the *PICALM* variant in SAS did not convince us,

we performed a replication study in an independent cohort of dogs. We used samples from 399 Newfoundland dogs, of which 102 had been examined by specialized veterinary cardiologists during the routine screening program of the German and Swiss Newfoundland clubs. We grouped the Newfoundland dogs into five different phenotype classes: (A) dogs with unknown phenotype ( $n = 286$ ); (B) dogs that were found to be SAS non-affected by a veterinarian, based upon negative auscultation findings (without a murmur), but without a documented measurement of maximal aortic outflow tract velocity (LVOT  $V_{\max}$ ;  $n = 11$ ); (C) dogs classified as SAS non-affected with an LVOT  $V_{\max} < 1.9$  m/s ( $n = 88$ ); (D) dogs classified as SAS equivocal with an LVOT  $V_{\max} = 1.9$ – $2.4$  m/s ( $n = 8$ ) and (E) dogs classified as SAS affected with an LVOT  $V_{\max} > 2.4$  m/s ( $n = 6$ ). The classes C, D and E were formed with the same criteria as used in the study by Stern et al. We additionally used samples from 500 dogs of 64 diverse other breeds without detailed information on their SAS phenotypes. Samples from these dogs were submitted to our research laboratory in the course of other research projects and we assumed them to be free of SAS. We designed PCR primers and amplified and sequenced the region containing the *PICALM*:p.K599\_L600insL variant from all 899 dogs.

The *PICALM* insertion had an allele frequency of 0.39 in our cohort of 399 Newfoundland dogs and it occurred in either heterozygous or homozygous state in 259 (65 %) of our Newfoundland dogs. Stern et al. state a dominant mode of action with a penetrance of 80.6 % for this allele. This means that 209 dogs or 52 % of our cohort would have been expected to be affected by SAS. In contrast to these estimations we were aware of only 14 either SAS-affected or SAS equivocal dogs, which represent less than 14 % of the 102 dogs in our cohort that underwent a cardiac exam. The SAS prevalence in the general Newfoundland population has

**Table 1** *PICALM*:p.K599\_L600insL allele and genotype distribution in Newfoundland dogs

References	Phenotype class	<i>n</i>	Allele distribution		Genotype distribution		
			wt	ins	wt/wt	wt/ins	ins/ins
Stern et al. (2014)							
	SAS non-affected	23	39 (0.85)	7 (0.15)	17 (0.74)	5 (0.22)	1 (0.04)
	SAS affected	26	18 (0.35)	34 (0.65)	1 (0.04)	16 (0.62)	9 (0.35)
This study							
	Phenotype unknown	286	344 (0.60)	228 (0.40)	98 (0.34)	148 (0.52)	40 (0.14)
	SAS non-affected, LVOT $V_{\max}$ unknown	11	15 (0.68)	7 (0.32)	6 (0.55)	3 (0.27)	2 (0.18)
	SAS non-affected, LVOT $V_{\max} < 1.9$ m/s	88	106 (0.60)	70 (0.40)	30 (0.34)	46 (0.52)	12 (0.14)
	SAS equivocal, LVOT $V_{\max} = 1.9$ – $2.4$ m/s	8	11 (0.69)	5 (0.31)	4 (0.50)	3 (0.38)	1 (0.13)
	SAS affected, LVOT $V_{\max} > 2.4$ m/s	6	8 (0.67)	4 (0.33)	2 (0.33)	4 (0.67)	0 (0.00)

While Stern et al. reported a significant association of the insertion with SAS in their cohort, there is no statistically significant difference between the allele or genotype distribution in any of the five phenotype classes investigated in this study ( $p_{\text{allelic}} = 0.91$ ;  $p_{\text{genotypic}} = 0.80$ ; Fisher’s exact test)

been reported to be less than 5 % based on more than 6,000 analyzed dogs (Reist-Marti et al. 2012).

In our replication study, we neither found an allelic nor a genotypic association for the PICALM:p.K599\_L600insL variant with the SAS phenotype (Table 1). Finally, we observed the insertion allele also in other breeds. In a cohort of 500 dogs from 64 breeds, 25 dogs were heterozygous for this variant (Table S1).

In conclusion, our data strongly suggest that the PICALM:p.K599\_L600insL variant is not causally involved in the development of SAS in Newfoundland dogs, but rather a neutral variant that occurs at high frequencies in Newfoundland dogs and is rare or absent in most other dog breeds. Consequently, the genotype at this variant is not a meaningful predictor of SAS risk. Rather, use of the commercially available genetic test has the potential to cause great harm as 65 % of the Newfoundland breed would be considered at risk for SAS and the sudden exclusion of such a large proportion of the breeding population would have a severe impact on the genetic diversity

of the breed and potentially detrimental consequences regarding other (recessive) genetic defects.

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**Conflict of interest** The authors are scientific competitors of the investigators of the original study. The authors have no financial conflict of interest to declare.

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